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Original article

Intracellular Ca^{2+}- and PKC-dependent upregulation of T-type Ca^{2+} channels in LPC-stimulated cardiomyocytes

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ABSTRACT

Lysophosphatidylcholine (LPC) accumulation in intracellular and/or interstitial space in cardiomyocytes may underlie as a mechanism for tachycardia and various arrhythmias during cardiac ischemia, which is usually accompanied by elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The present study was therefore designed to investigate possible mechanisms responsible for $[Ca^{2+}]_i$ elevation by LPC focusing on T-type Ca^{2+} channel current ($I_{Ca.T}$). LPC as well as phorbol 12-myristate 13-acetate (PMA) significantly accelerated the beating rates of neonatal rat cardiomyocytes. Augmentation of $I_{Ca.T}$ by LPC was dependent on the intracellular Ca^{2+} concentration: an increase of $I_{Ca.T}$ was significantly larger in high $[Ca^{2+}]_i$ condition (pCa = 7) than those in low $[Ca^{2+}]_i$ condition (pCa = 11). In heterologous expression system by use of human cardiac $Ca_V3.1$ and $Ca_V3.2$ channels expressed in HEK293 cells, LPC augmented $Ca_V3.2$ channel current ($I_{Cav3.2}$) in a concentration-dependent manner but not $Ca_V3.1$ channel current ($I_{Cav3.1}$). Augmentation of $I_{Cav3.2}$ by LPC was highly $[Ca^{2+}]_i$ dependent: $I_{Cav3.2}$ was unchanged when pCa was 11 but was markedly increased when $[Ca^{2+}]_i$ was higher than 10^{-10} M (pCa ≤ 10) by LPC application (10–50 μM). A specific inhibitor of protein kinase Cα (Ro-32-0432) attenuated the increase of $I_{Cav3.2}$ by LPC. LPC stimulates $I_{Ca.T}$ in a $[Ca^{2+}]_i$-dependent manner via PKCα activation, which may play a role in triggering arrhythmias in pathophysiological conditions of the heart.

1. Introduction

Lysophosphatidylcholine (LPC) is an amphiphilic phospholipid present in small quantities (0.5–3.5% of the total phospholipids) in the myocardium under physiological conditions [1,2]. However, in ischemic conditions, LPC accumulates promptly by 50% or more, mainly due to activation of phospholipase A_2 [2,3]. The increase of LPC depolarizes the membrane potentials and evokes electric disturbance as well as mechanical defect in the heart [3,4]. LPC accumulation in intracellular and/or interstitial space in cardiomyocytes may underlie as a mechanism for tachycardia and various arrhythmias during cardiac ischemia [4]. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) elevation caused by LPC accumulation plays an important role in triggering onset of arrhythmias in cardiac ischemia [1,2,4,5]. The several possible mechanisms responsible for $[Ca^{2+}]_i$ elevation by LPC are postulated: an increase in membrane permeability to Ca^{2+} [3], an inhibition of Na^+-K^+-ATPase activity [5] and activation of the Na^+-Ca^{2+} exchanger [1]. In the previous study, we found that LPC augments the T-type Ca^{2+} channel current ($I_{Ca.T}$) in heterologous system [6]. However, the underlying intracellular signal mechanisms are mostly unknown.

Protein kinase C (PKC) is an enzyme in signal transduction involved in a variety of cellular functions and has been identified as a key molecule in case of various pathological conditions in the heart [7]. Three PKC subgroups have been identified: (1) conventional PKC (α, βI, βII and γ) which are activated by phosphatidylserine, intracellular Ca^{2+} and diacylglycerol (DAG); (2) novel PKC (δ, ε, η and θ) which are activated by DAG but not intracellular Ca^{2+}; and (3) atypical PKC (ζ and λ/ι) which are not activated by intracellular Ca^{2+} or DAG [7]. Out of the 11 PKC isoforms, α, βI, βII, δ, ε, ζ and λ have been identified in the heart [8-10]. PKC may phosphorylate a myriad of target proteins including ion channels, thereby regulating cellular excitation processes [11]. The present study therefore was designed to investigate the mechanisms responsible for $I_{Ca.T}$ modulation by LPC by means of PKC isoform activation using the whole-cell patch-clamp technique in neonatal rat cardiomyocyte and heterologous expression system using human $Ca_V3.1$ and $Ca_V3.2$ T-type Ca^{2+} channels. Our results indicate that PKCα activation by LPC upregulates the $Ca_V3.2$ T-type Ca^{2+} channel in a $[Ca^{2+}]_i$-dependent manner, suggesting a novel mechanism for tachyarrhythmias in ischemic heart diseases.

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2. Materials and methods

2.1. Rat cardiomyocytes and $Ca_V3.1$/$Ca_V3.2$-HEK293 cells culture

The experimental protocol was approved in advance by Ethics Review Committee for Animal Experimentation of Oita University, and confirms with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH publication No.85-23, revised 1996). Neonatal rat cardiomyocytes from 1- to 3-day-old Wistar rats [12] and hypertrophied ventricular myocytes from Wistar rats were prepared as described previously [13]. In brief, a single dose of 60 mg/kg monocrotaline was injected into the intraperitoneal cavity at the age of 8 weeks old, and right ventricular myocytes were isolated enzymatically at the age of 14 weeks from male Wistar rats. Human cardiac T-type Ca^{2+} channel α1 subunits, $Ca_V3.1$ and $Ca_V3.2$, were stably expressed in HEK293 cells ($Ca_V3.1$-HEK293 and $Ca_V3.2$-HEK293, respectively) as described in our previous report [6].

2.2. Whole-cell current recordings

Macroscopic L-type Ca^{2+} channel currents ($I_{Ca.L}$) and T-type Ca^{2+} channel currents ($I_{Ca.T}$) were recorded at room temperature (20–23 °C) by voltage-clamp technique as described previously [6,12,13]. Action potentials of neonatal rat cardiomyocytes were recorded by current-clamp technique [12]. Dose–response curves were obtained by the equation as follows: $\text{fraction} = I_{min} + (I_{max} - I_{min})/\{1 + ([C]/EC_{50})^h\}$, where I_{min} is the minimum $I_{Ca.T}$, I_{max} is the maximum $I_{Ca.T}$ after LPC application, [C] is the concentration of LPC, EC_{50} is the half-maximal effecting concentration of $I_{Ca.T}$ by LPC and *h* is the Hill slope.

2.3. Solutions and chemicals

For action potential recordings, the bath solution (Tyrode's solution) contained (in mM) NaCl 140, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 1.8 and glucose 10 (pH was adjusted to 7.4 with 1N NaOH), and the pipette solution contained (in mM) KCl 140, NaCl 5, MgATP 5, Na_2 ATP 5 and EGTA 0.05 (pH was adjusted to 7.2 with 1N KOH) (solution E in Table 1). For $I_{Ca.T}$ recording, the bath solution contained (in mM) tetraethylammonium chloride (TEA-Cl) 120, CsCl 6, $MgCl_2$ 0.5, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) 0.1, 4-amino-pyridine (4-AP) 5, HEPES 10, $CaCl_2$ 1.8, glucose 10 and tetrodotoxin (TTX) 0.01 (pH was adjusted to 7.4 with 1N TEA-OH), and five different pipette solutions were prepared by means of different Ca^{2+} concentrations according to the equation by Fabiato [14] together with (in mM) CsCl 130, $MgCl_2$ 2, ATP-Mg 2, GTP-Na 0.5 and HEPES 5, where pH was adjusted to 7.2 with 1N CsOH (solutions A, B, C, D and F in Table 1).

A PKC activator PMA (phorbol 12-myristate 13-acetate), a pan-protein kinase C (PKC) inhibitor chelerythrine (1,2-dimethoxy-*N*-methyl(1,3)benzodioxolo(5,6-c)phenanthridinium chloride), a PKCα inhibitor Ro-32-0432 (3-(8-((dimethylamino)methyl)-6,7,8,9-tetrahydropyrido [1,2-*a*]indol-10-y1)-4-(1-methyl-1*H*-indol-3-y1)-1*H*-pyrrole-2,5-dione hydrochloride), a PKCβI inhibitor Gö 6976 (12-(2-cyanoethyl)-6,7,12,13-tetrahydro- 13-methyl-5-oxo-5*H*-indolo(2,3-*a*) pyrrolo(3,4-*c*)-carbazole) and a PKCβII inhibitor CGP-53353 (5,6-bis [(4-fluorophenyl)amino]-2*H*-isoindole-1,3-dione) were purchased from Calbiochem Co. (La Jolla, CA). These protein kinase inhibitors were applied at the concentration of 5–10 times the ones inducing a 50% inhibition (IC_{50}) of each protein kinase [15-18]. These protein kinase inhibitors were dissolved in dimethyl sulfoxide (DMSO), where the final concentration of DMSO was less than 0.01%.

Table 1
Calculated Ca^{2+} concentration in pipette solution ($[Ca^{2+}]_i$).

Solution	[EGTA], mM	[$CaCl_2$], mM	$[Ca^{2+}]_p$, mM	pCa
A	40	0	10^{-11}	11
B	10	0	10^{-10}	10
C	0.2	0	10^{-9}	9
D	0.386	0.1	10^{-8}	8
E	0.05	0	$10^{-7.2}$	7.2
F	0.2	0.152	10^{-7}	7

[EGTA] and [$CaCl_2$] in pipette solutions used to vary $[Ca^{2+}]_i$. All compositions were calculated using program provided by Fabiato [14].

2.4. Data acquisition and statistical analysis

The data were acquired by using computer software (Pulse/PulseFit, V.8.11, HEKA Elektronik). The group data are shown as means ± SD. Analysis of variance and Tukey–Kramer procedure were used for multiple comparisons, and Student's *t* test was used for the comparison of two groups. Differences were considered significant when *p* values were less than 0.05.

3. Results

3.1. Lysophosphatidylcholine (LPC) accelerates the cardiomyocyte automaticity

LPC markedly accelerated the spontaneous beating rates of neonatal rat cardiomyocytes from 42 ± 8 bpm in control to 64 ± 8 bpm after LPC application in 5 min (Figs. 1A and C), at the physiological $[Ca^{2+}]_i$ condition (pCa = 7.2). However, the maximum upstroke velocity $\dot{V}_{max}$ (3.0 ± 0.9 V/s, control vs. 3.1 ± 0.8 V/s, LPC) and the maximum diastolic potential (MDP) (−59.8 ± 1.6 mV, control vs. −53.6 ± 1.7 mV, LPC) were unchanged (Figs. 1D and E). Meanwhile, application of PMA accelerated the spontaneous beating rate from 42 ± 8 bpm to 78 ± 9 bpm in 4 min (Figs. 1B and F). $\dot{V}_{max}$ was significantly increased by PMA (2.8 ± 0.8 V/s, control vs. 4.7 ± 1.0 V/s, PMA) (Fig. 1G), which unfolded the different influence compared with the effect of LPC. MDP was unaltered (−59.8 ± 1.6 mV, control vs. −59.5 ± 1.0 mV, PMA) by PMA application (Fig. 1H).

3.2. LPC augments $I_{Ca.T}$ in cardiomyocytes

We recorded L-type Ca^{2+} channel currents ($I_{Ca.L}$) and T-type Ca^{2+} channel currents ($I_{Ca.T}$) in single beating cardiomyocytes isolated from 1- to 3-day-old Wistar rats. Ca^{2+} channel currents were elicited by depolarizing the membrane to a test potential of −20 mV from a pair of holding potentials of −100 mV and −40 mV in order to discriminate $I_{Ca.L}$ and $I_{Ca.T}$ from whole Ca^{2+} channel current (I_{Ca}). To investigate effects of intracellular Ca^{2+} on $I_{Ca.L}$ and $I_{Ca.T}$ by LPC application, we prepared two different pipette solutions with different Ca^{2+} concentration at a pCa of 7 (solution F) and a pCa of 11 (solution A). In neonatal cardiomyocytes, $I_{Ca.T}$ was significantly increased by 10 μM LPC by 21.5% when $[Ca^{2+}]_i$ was high (pCa = 7) (Fig. 2A). However, $I_{Ca.T}$ was unchanged by LPC when $[Ca^{2+}]_i$ was low (pCa = 11) (Fig. 2B). On the other hand, $I_{Ca.L}$ was not changed by LPC either in high $[Ca^{2+}]_i$ condition of pCa = 7 or in low $[Ca^{2+}]_i$ condition of pCa = 11 (Figs. 2A and B). Intracellular Ca^{2+}-dependent augmentation of $I_{Ca.T}$ by LPC was confirmed not only in neonatal cardiomyocytes but in adult ventricular myocytes from the hypertrophied heart (Figs. 2C and D). Although the T-type Ca^{2+} channel is reportedly absent in adult normal cardiac ventricular myocytes, hypertrophied right ventricular myocytes produced by MCT injection are rich in the T-type Ca^{2+} channels as we have previously reported [13], and in this experiment. $I_{Ca.T}$ was significantly increased by 10 μM LPC by 23.5% when $[Ca^{2+}]_i$ was high (pCa = 7) (Fig. 2C), although it was unchanged when $[Ca^{2+}]_i$ was low (pCa = 11) (Fig. 2D). These data indicate that intracellular Ca^{2+} acts as an important factor to modulate $I_{Ca.T}$ by LPC.

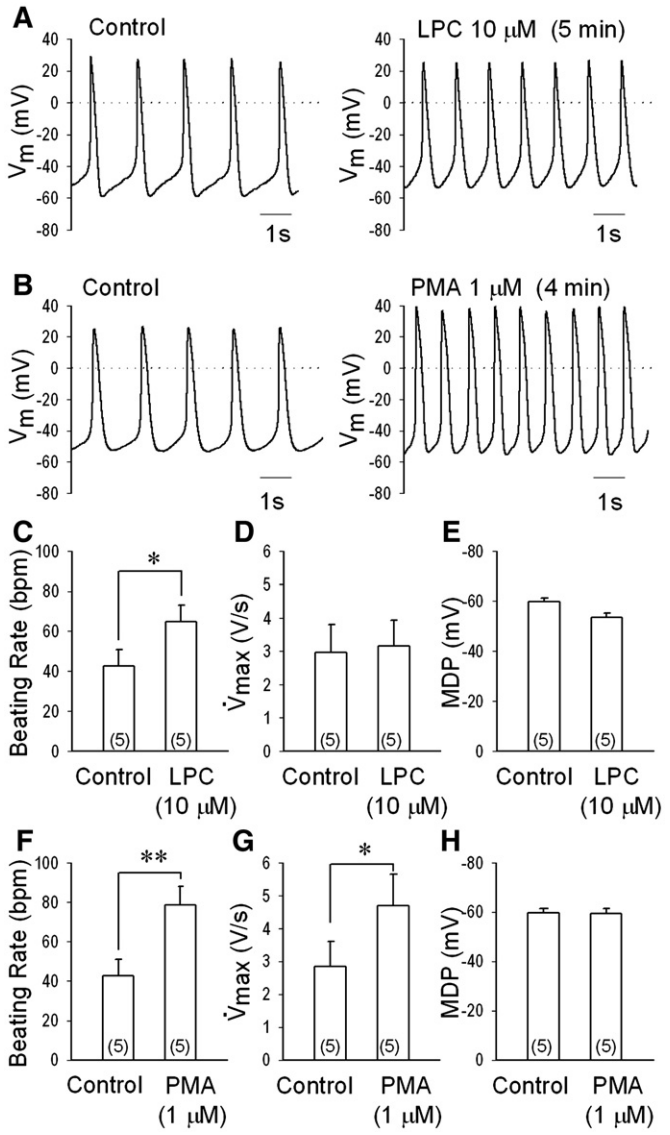

Fig. 1. LPC (10 μM) and PMA (1 μM) enhance the automaticity in spontaneous beating cardiomyocytes. Representative action potentials (AP) of neonatal rat cardiomyocyte recorded in control condition with LPC (10 μM) application in 5 min (A), and in control condition with PMA (1 μM) application in 4 min (B). Illustration of LPC (10 μM) effects on the beating rate (C), the maximum upstroke velocity (V_{max}) (D) and the maximum diastolic potential (MDP) (E). Illustration of PMA (1 μM) effects on the beating rate (F), V_{max} (G) and MDP (H). The number of cells used in each group is indicated in parentheses. *$p<0.05$, **$p<0.01$.

3.3. $[Ca^{2+}]_i$-dependent $I_{Ca.T}$ modulation by LPC

To further clarify effects of LPC on $I_{Ca.T}$ in terms of T-type Ca^{2+} channel isoform dependency, we focused on subtypes of the channel, $Ca_V3.1$ and $Ca_V3.2$, two major T-type Ca^{2+} channel pore-forming subunits identified in the heart. To this end, we utilized stably transfected HEK293 cell lines of $Ca_V3.1$ and $Ca_V3.2$ ($Ca_V3.1$-HEK293 and $Ca_V3.2$-HEK293).

LPC exerted no effect on the $Ca_V3.1$ T-type Ca^{2+} channel current ($I_{Cav3.1}$) regardless of the $[Ca^{2+}]_i$ condition at a pCa of 7 (solution F) or at a pCa of 11 (solution A) as shown in Figs. 3A and C. In contrast, LPC upregulated the $Ca_V3.2$ T-type Ca^{2+} channel current ($I_{Cav3.2}$), which was much larger at a pCa of 7 than that at a pCa of 11 (Figs. 3B and D). $I_{Cav3.2}$ was increased in a pCa-dependent manner by application of LPC, which was also concentration dependent on LPC of 10 and 50 μM. Importantly, LPC of 10 and 50 μM did not increase $I_{Cav3.2}$ when intracellular Ca^{2+} concentration was very low (pCa = 11) (Figs. 3E and F). These data indicate that LPC modulates $I_{Cav3.2}$ but not $I_{Cav3.1}$ by a mechanism in which intracellular Ca^{2+} is involved.

We explored efficacy and potency of LPC on $I_{Cav3.2}$ and $I_{Cav3.1}$ modulation in heterologous system. LPC markedly increased $I_{Cav3.2}$ in a high $[Ca^{2+}]_i$ condition. LPC modulates $I_{Cav3.2}$ with the E_{max} of 2.14 at pCa of 7, in contrast to the E_{max} of 1.05 at pCa of 11 (Fig. 3G). On the other hand, EC_{50} values for LPC at the different pCa conditions (pCa of 7–11) were nearly identical at ~8 μM (7.1–8.9 μM) as indicated in Fig. 3H. Efficacy of LPC on $I_{Cav3.2}$ is stable in the pCa range of 7–9, indicating that $I_{Ca.T}$ modulation by LPC is solely dependent on LPC concentration unless intracellular Ca^{2+} concentration is subphysiologically low. Ineffectiveness of LPC on $I_{Cav3.1}$ was reconfirmed at the pCa of 7–11 (Fig. 3I).

3.4. $I_{Ca.T}$ modulation by LPC is mediated by PKC activation

Possible involvement of PKC activity in LPC actions on $I_{Ca.T}$ upregulation was investigated in this context. $Ca_V3.2$-HEK293 cells were pretreated with PKC inhibitors chelerythrine, Ro-32-0432, Gö 6976 or CGP-53353 to inhibit whole PKC, PKCα, PKCβI or PKCβII, respectively. In Fig. 4, LPC did not augment $I_{Cav3.2}$ with a pretreatment using chelerythrine, firmly suggesting that an increase of $I_{Cav3.2}$ by LPC was caused by PKC activation. A specific PKCα inhibitor Ro-32-0432 completely blocked the effect of LPC on $I_{Cav3.2}$. However, in the same culture condition, a specific PKCβI inhibitor Gö 6976 (20 nM) and a specific PKCβII inhibitor CGP-53353 (2 μM) did not modify the effect of LPC on $I_{Cav3.2}$.

3.5. Interaction of PMA and LPC on $I_{Ca.T}$

In Fig. 1, LPC as well as PMA increased the spontaneous beating rate in cardiomyocytes in a similar fashion. We therefore examined the interaction of LPC and PKC actions on $I_{Ca.T}$ modulation (Fig. 5). PMA treatment of $Ca_V3.2$-HEK293 cells for 1 h upregulated $I_{Cav3.2}$ by 51.0 ± 1.6% ($n=5$) at the level of maximum peak current (Figs. 5C and D). Importantly, the channel kinetics were unchanged by PMA; half activation potential ($V_{1/2}$) was −46.5 ± 2.1 mV ($n=5$) without PMA (control) and −47.6 ± 1.8 mV ($n=5$) with PMA (Fig. 5E); time constant (τ) for the current decay (fast inactivation) at the potential of −30 mV (the potential for the maximum inward current) was 26.4 ± 2.7 ms ($n=5$) without PMA (control) and 28.0 ± 0.8 ms ($n=5$) with PMA (Fig. 5F). We found that $I_{Cav3.2}$ was not changed by LPC (50 μM) when $Ca_V3.2$-HEK293 cells were fully activated by PKC with a very high concentration of PMA (1 μM) (Figs. 5A, C, and D). The maximum PKC activation by PMA masked the effect of LPC on $I_{Cav3.2}$ (Figs. 5A, C, and G). On the other hand, when $I_{Cav3.2}$ was maximally activated by 20 μM LPC based on the results in Figs. 3G and H, PMA of 1 μM further augmented $I_{Cav3.2}$ (Fig. 5B). Our data therefore confirmed that LPC-induced $I_{Cav3.2}$ modulation was mediated by PKCα activation.

We also focused on exploring the effect of PMA on $I_{Cav3.1}$ (Fig. 6). As shown in Figs. 6A and D, unlikely to the effect of LPC, PMA (1 μM) increased the $I_{Cav3.1}$ by 60.9 ± 2.2% ($n=6$) at the level of maximum peak current in the same experimental condition as shown in Fig. 5A and B. $I_{Cav3.1}$ kinetics were unchanged by PMA; the $V_{1/2}$ of activation was −42.7 ± 1.9 mV ($n=5$) without PMA (control) and −42.0 ± 2.1 mV ($n=6$) with PMA (Fig. 6E); τ for the current decay (fast inactivation) at the potential of −30 mV was 20.4 ± 2.1 ms ($n=5$) without PMA (control) and 18.6 ± 2.2 ms ($n=6$) with PMA (Fig. 6F), which is highly comparable to those of PMA on $I_{Cav3.2}$ (Fig. 5). We found that $I_{Cav3.1}$ was not changed by 10–50 μM LPC, when $Ca_V3.1$-HEK293 cells were pretreated by PMA (1 μM) for 1 h (Fig. 6A, C, and D). The maximum PKC activation by PMA (1 μM) masked the effect of LPC on $I_{Cav3.1}$ (Fig. 6G). Interestingly, $I_{Cav3.1}$, unchanged by 20 μM LPC (Fig. 6B) or 50 μM LPC (not shown), was markedly increased by PMA (1 μM), which revealed a distinction between $I_{Cav3.1}$ and $I_{Cav3.2}$ modulation by LPC and PKC.

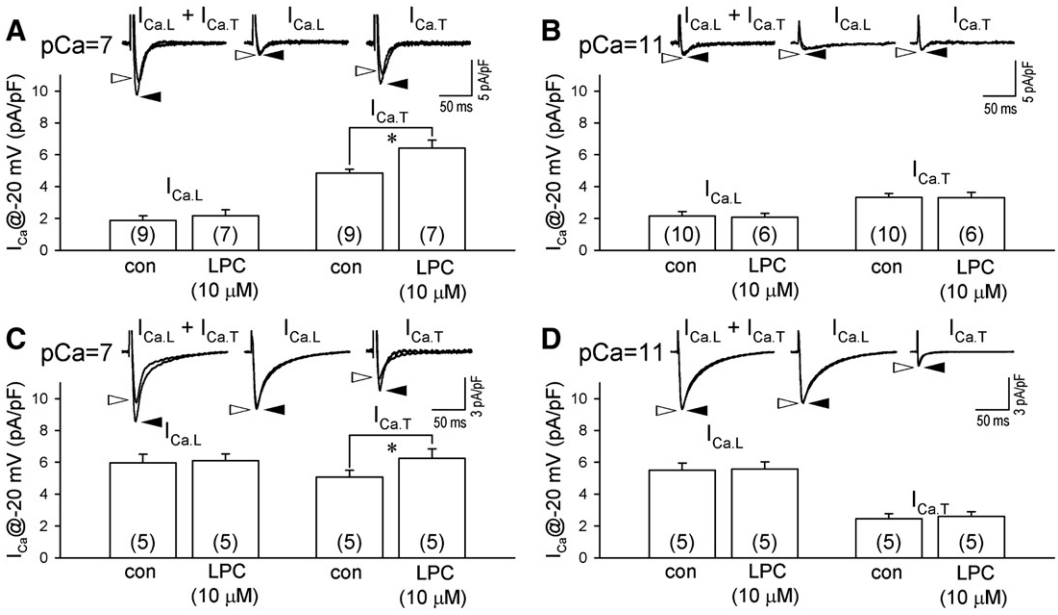

Fig. 2. Effects of LPC on $I_{Ca.L}$ and $I_{Ca.T}$ in neonatal rat cardiomyocytes and in adult rat ventricular myocytes with different $[Ca^{2+}]_i$ condition. Representative current traces of total I_{Ca} ($I_{Ca.L}+I_{Ca.T}$), $I_{Ca.L}$ and $I_{Ca.T}$ with ( ) or without 10 μM LPC (▷), and group data for $I_{Ca.L}$ and $I_{Ca.T}$ at the membrane potentials of −20 mV normalized to the cell capacitance with or without LPC (10 μM) at a pCa of 7 in neonatal myocytes (A) and in adult right ventricular myocytes (C), and at a pCa of 11 in neonatal myocytes (B) and in adult right ventricular myocytes (D). The number of cells used in each group is indicated in parentheses. *$p<0.05$.

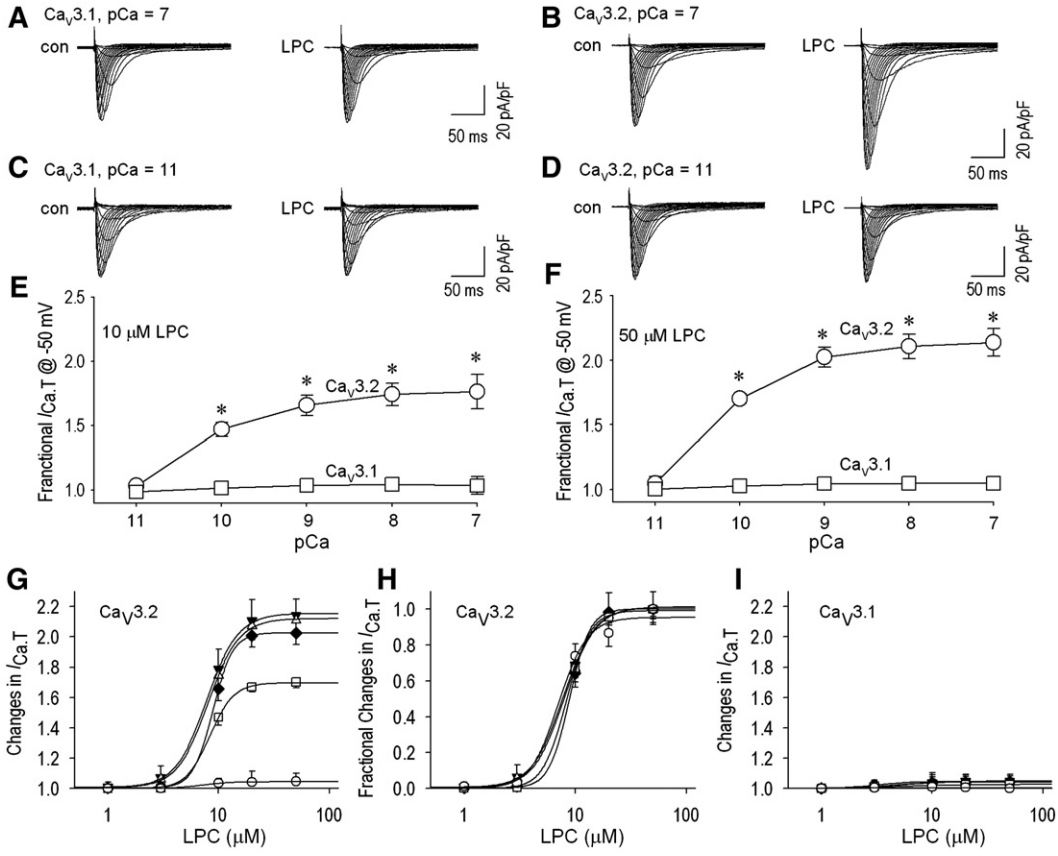

Fig. 3. Effects of LPC (10 μM) on two subtypes of human cardiac T-type Ca^{2+} channel α subunit currents, $I_{Cav3.1}$ and $I_{Cav3.2}$. $I_{Cav3.1}$ family traces in the control condition and in LPC (10 μM) application in 5 min recorded in high $[Ca^{2+}]_i$ condition (pCa = 7) (A) and in a low $[Ca^{2+}]_i$ condition (pCa = 11) (C). $I_{Cav3.2}$ family traces in the control condition and LPC (10 μM) application in 5 min recorded in high $[Ca^{2+}]_i$ condition (pCa = 7) (B) and in a low $[Ca^{2+}]_i$ condition (pCa = 11) (D). Intracellular Ca^{2+} concentration-dependent effects of 10 μM LPC (E) and 50 μM LPC (F) on $I_{Cav3.1}$ and $I_{Cav3.2}$. $I_{Ca.T}$, $I_{Cav3.1}$ and $I_{Cav3.2}$, with LPC application in 5–10 min at the membrane potentials of −50 mV, were evaluated as a fraction normalized to ones without LPC. Dose-dependent changes of $I_{Cav3.2}$ and $I_{Cav3.1}$ by LPC were assessed by different pCa conditions (●, pCa = 11; ■, pCa = 10; ◆, pCa = 9; ▲, pCa = 8; ▼, pCa = 7). E_{max} of LPC for $I_{Cav3.2}$ modulation was 1.05, 1.70, 2.02, 2.11 and 2.14 at pCa of 11, 10, 9, 8 and 7, respectively (G). Changes in $I_{Ca.T}$ (G) were normalized to each E_{max} at different pCa conditions yielding EC_{50} for 7.1, 8.3, 8.9, 8.0 and 7.8 μM at the pCa of 11, 10, 9, 8 and 7, respectively (H). Dose-dependent $I_{Cav3.1}$ change by LPC was assessed by different pCa conditions (I). Data were fitted by the Hill equation as described in Materials and methods.

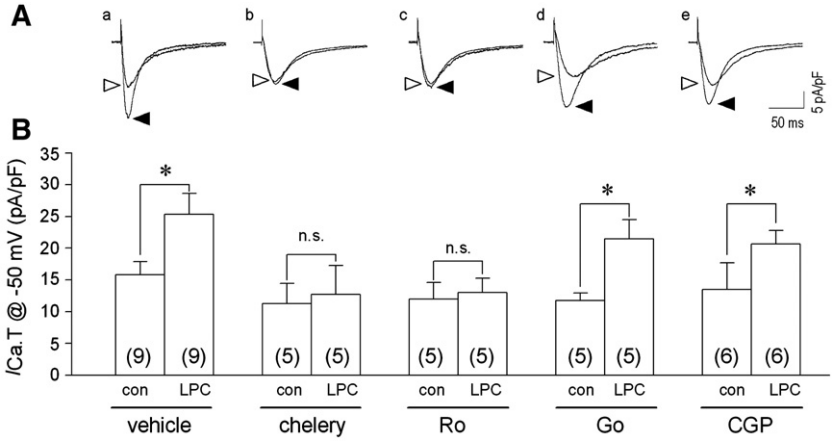

Fig. 4. Effects of PKC inhibitors on the modulation of $I_{Cav3.2}$ by LPC. (A) Representative $I_{Cav3.2}$ traces at the potential of −50 mV with ( ) or without (▷) 10 μM LPC in the presence of vehicle (a), a pan-PKC inhibitor (chelery: chelerythrine, 5 μM) (b), a specific PKCα inhibitor (Ro: Ro-32-0432, 30 nM) (c), a specific PKCβI inhibitor (Go: Gö 6976, 20 nM) (d) and a specific PKCβII inhibitor (CGP: CGP-53353, 2 μM) (e). (B) Group data for LPC effects on $I_{Ca.T}$ ($I_{Cav3.2}$) at the membrane potentials of −50 mV with or without PKC isoform inhibitors. The number of cells used in each group is indicated in parentheses. *$p<0.05$.

3.6. Impacts of PMA and LPC on Ni^{2+}-resistant AP

In order to further confirm the difference between LPC and PMA on cardiomyocyte automaticity, we have recorded AP with low concentration of Ni^{2+} in the bath solution. T-type Ca^{2+} channel isoforms are reportedly characterized by the properties of their block by Ni^{2+}; $I_{Cav3.2}$ is more than 10 times sensitive to Ni^{2+} than $I_{Cav3.1}$. In our experimental conditions by use of heterologous expression of human $Ca_V3.1$ and $Ca_V3.2$ in HEK cells, 50 μM Ni^{2+} reduced $I_{Cav3.1}$ by ~15% and $I_{Cav3.2}$ by ~95% (Fig. 7A). On the basis of these results, beating rates were approximately halved by 50 μM Ni^{2+} (Figs. 7D and G). In the presence of 50 μM Ni^{2+}, LPC (10 μM) failed to accelerate the spontaneous beating rates of neonatal rat cardiomyocytes (Fig. 7D), supporting our results that LPC has no effects on $I_{Cav3.1}$ or Ni^{2+}-insensitive $I_{Ca.T}$ as shown in Figs. 1 and 6. In contrast, in the presence of 50 μM Ni^{2+}, PMA (1 μM) significantly increased the beating rates from 18 ± 3 bpm to 28 ± 6 bpm (Fig. 7G), reflecting the ability of PMA to increase $I_{Cav3.1}$ (Ni^{2+}-insensitive $I_{Ca.T}$) as well as $I_{Cav3.2}$ (Ni^{2+}-sensitive

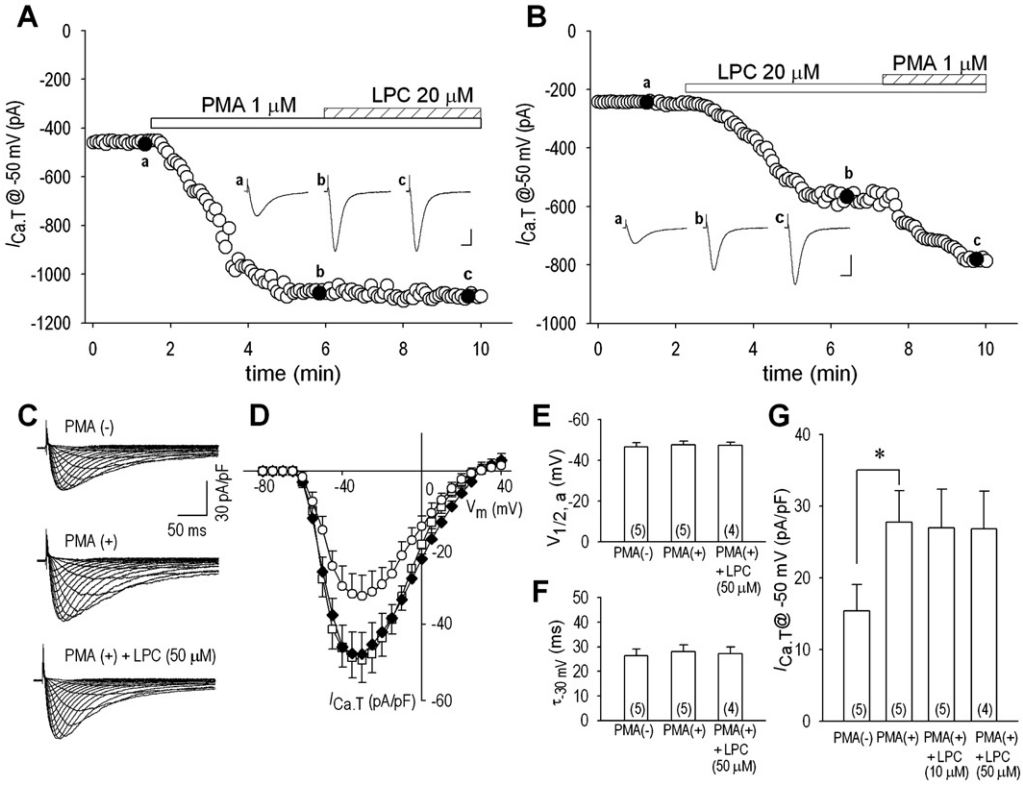

Fig. 5. Interaction of PMA and LPC effects on $I_{Cav3.2}$. $I_{Ca.T}$ ($I_{Cav3.2}$) family current traces recorded in $Ca_V3.2$-HEK293 cells without PMA pretreatment (PMA (−)), with PMA (1 μM) pretreatment for 1 h (PMA (+)) and PMA (+) with LPC (50 μM) application in 5 min (A). (B) The current–voltage relationships with PMA (−) (○), PMA (+) (●), and PMA (+) with LPC (50 μM) application (◆). Half activation potentials ($V_{1/2}$) (C), time constants (τ) for the current decay at the potential of -30 mV (D) and the maximum peak current at the potentials of −50 mV (E) with PMA (−), PMA (+), PMA (+) with LPC of 10 μM and PMA (+) with LPC of 50 μM. (F) Representative $I_{Ca.T}$ recorded at the membrane potentials of −50 mV under the effects of PMA (1 μM) alone and combined with LPC (20 μM) application. (G) Representative $I_{Ca.T}$ recorded at the membrane potentials of −50 mV under the effects of LPC (20 μM) application alone and combined with PMA (1 μM). Current traces in insets (a–c) were recorded at the points indicated (●) in (F) and (G), respectively. Scale bars for 50 ms and 20 pA/pF in insets in (F) and (G). The number of cells used in each group is indicated in parentheses. *$p<0.05$.

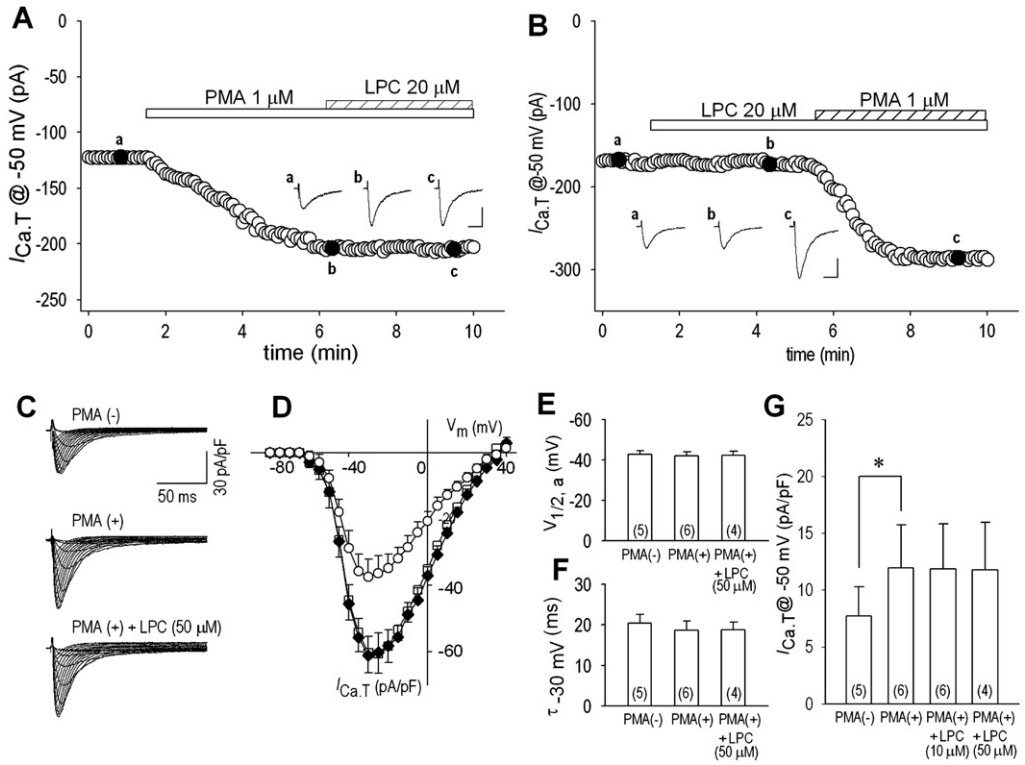

Fig. 6. Interaction of PMA and LPC effects on $I_{Cav3.1}$. $I_{Ca.T}$ ($I_{Cav3.1}$) family current traces recorded in $Ca_V3.1$-HEK293 cells without PMA pretreatment (PMA (−)), with PMA (1 μM) pretreatment for 1 h (PMA (+)) and PMA (+) with LPC (50 μM) application in 5 min (A). (B) The current–voltage relationships with PMA (−) (○), PMA (+) (●) and PMA (+) with LPC (50 μM) application (◆). Half activation potentials ($V_{1/2}$) (C), time constants for the current decay at the potential of −30 mV (D) and the maximum peak current at the potentials of −50 mV (E) with PMA (−), PMA (+), PMA (+) with LPC of 10 μM and PMA (+) with LPC of 50 μM. (F) Representative $I_{Ca.T}$ recorded at the membrane potentials of −50 mV under the effects of PMA (1 μM) alone and combined with LPC (20 μM) application. (G) Representative $I_{Ca.T}$ recorded at the membrane potentials of −50 mV under the effects of LPC (20 μM) application alone and following combined with PMA (1 μM). Current traces in insets (a–c) were recorded at the points indicated (●) in (F) and (G), respectively. Scale bars for 50 ms and 20 pA/pF in panels (F) and (G). The number of cells used in each group is indicated in parentheses. *$p<0.05$.

$I_{Ca.T}$) as demonstrated in Figs. 5 and 6. The maximum upstroke velocity ($\dot{V}_{max}$) and the maximum diastolic potential (MDP) were statistically unchanged by LPC and PMA in the presence of 50 μM Ni^{2+}.

4. Discussion

The present study demonstrates that LPC and PKC activation by phorbol ester accelerates automaticity in cardiac myocytes by enhancing $I_{Ca.T}$. Although a pair of reports previously demonstrated an enhancement of automaticity in cardiac Purkinje fibers by LPC [19,20], the present study has clarified the underlying mechanism of modulation in pacemaker potentials by LPC, revealing the T-type Ca^{2+} channel activation by PKCα.

4.1. LPC and PMA accelerate the automaticity of cardiomyocytes

LPC, an amphiphilic acid, accumulates in intracellular and extracellular space during cardiac ischemia and is important to generate arrhythmias by modulating various membrane currents. For example, LPC attenuates the K^+ channel conductance to produce membrane depolarization [21], and modulates Na^+ current as well [22]. Based on the findings that spontaneous beating rate of cardiomyocytes was accelerated by LPC, it is postulated that LPC regulates the membrane potentials by upregulating the pacemaker channels, including the L-type Ca^{2+} channel, the T-type Ca^{2+} channel and the hyperpolarization-activated inward cation channel. In the present study, we focused on the effects of LPC on $I_{Ca.L}$ and $I_{Ca.T}$. $I_{Ca.L}$ was not changed by LPC either in the high $[Ca^{2+}]_i$ condition (pCa = 7) or in the low $[Ca^{2+}]_i$ condition (pCa = 11) (Fig. 2). In the literatures, effects of LPC on $I_{Ca.L}$ are controversial; some researchers have shown an increase of Ca^{2+} influx through $I_{Ca.L}$ [2], and others have described the ineffectiveness of LPC on $I_{Ca.L}$ [1,4]. This discrepancy might be attributed to the differences of cell types or cellular conditions including $[Ca^{2+}]_i$. The present study clearly demonstrates that the effect of LPC on $I_{Ca.T}$ is highly dependent on $[Ca^{2+}]_i$. LPC (10 μM) markedly increases $I_{Ca.T}$ only when $[Ca^{2+}]_i$ is in the physiological condition (pCa ≤ 10), which is consistent with an enhancement of automaticity in spontaneous beating myocytes at pCa of 7.2 (Fig. 1). A hyperpolarization-activated inward current (I_f) is one of the pacemaker channel currents responsible for the generation of slow diastolic depolarization of action potentials in cardiomyocyte with automaticity [23]. Although we did not show any patch-clamp data on I_f in this study, no change of I_f by LPC (10–50 μM) was observed by use of HCN4 in heterologous system in HEK293 cells (data not shown). Because LPC may stimulate adenylate cyclase, possibly resulted in an increase in $[cAMP]_i$ [24], which may contribute to I_f augmentation, further precise studies focusing on I_f are obviously needed.

Importantly, application of PMA accelerated the beating rates of neonatal cardiomyocytes when intracellular Ca^{2+} concentration is in the physiological range at pCa of 7.2 (Figs. 1F and G). It is suggested that PMA, a PKC activator, may accelerate the pacemaker potentials via the same mechanism as does LPC. In this connection, it is of interest to compare the ability of LPC and PMA to increase the beating rate of cardiomyocytes. When the beating rate was nearly halved by 50 μM Ni^{2+}, it returned to approximately 75% of control levels by an additional application of PMA (Fig. 7G). Taking into account that in HEK293 cells 50 μM Ni^{2+} almost completely blocks $I_{Cav3.2}$, and that PMA has an ability to increase $I_{Cav3.1}$, $I_{Cav3.1}$ and $I_{Cav3.2}$ may contribute to the same extent to the generation of pacemaker potentials in neonatal cardiomyocytes. From this point of view, up-regulation of both $I_{Cav3.1}$ and $I_{Cav3.2}$ by PMA (Fig. 1F) is obviously more effective than that of $I_{Cav3.2}$ alone by PLC in accelerating the cellular

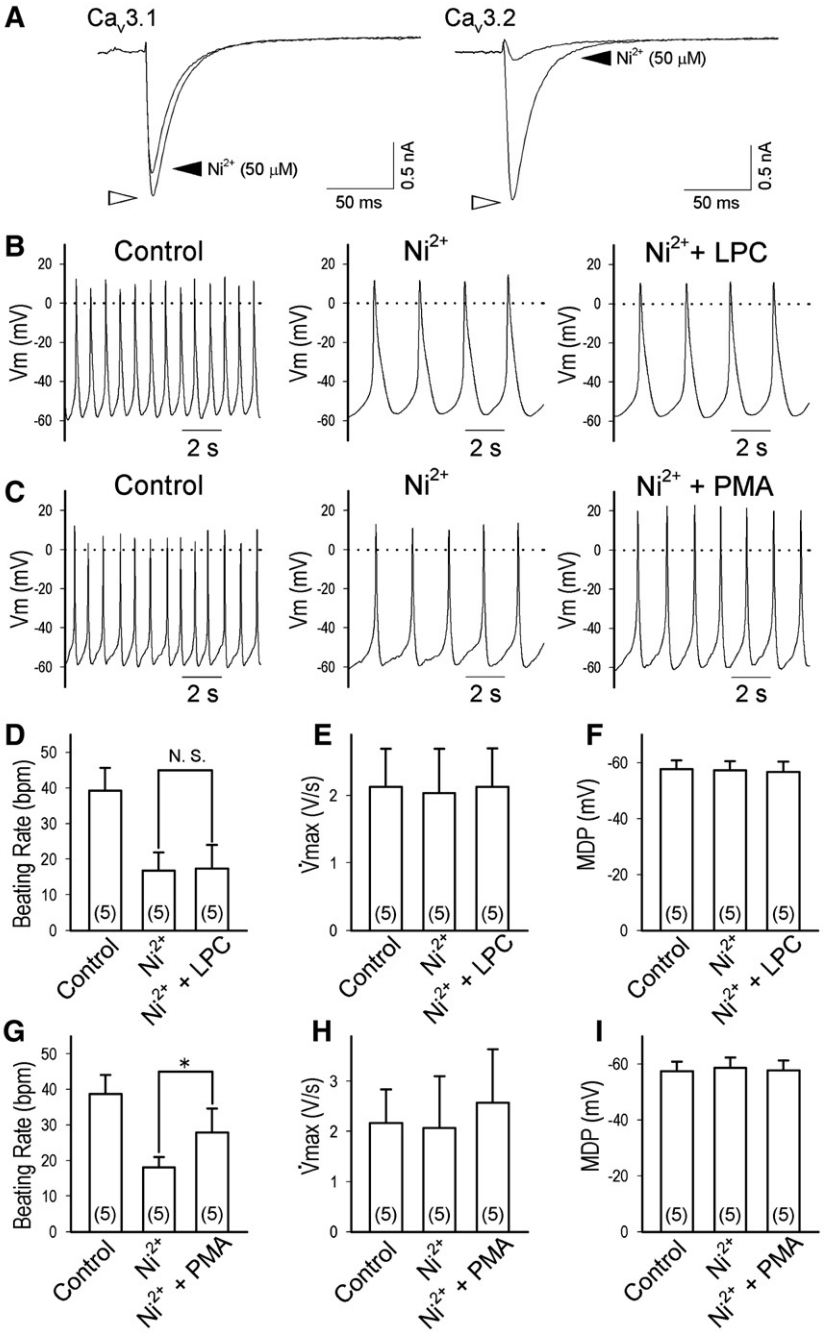

Fig. 7. PMA (1 μM) but not LPC (10 μM) enhances the automaticity in spontaneous beating cardiomyocytes when treated with low concentration of Ni^{2+}. Representative $I_{Cav3.1}$ and $I_{Cav3.2}$ current traces recorded at −30 mV from the holding potential of −100 mV before (hollow triangles) and after (filled triangles) application of 50 μM Ni^{2+} (A). Representative AP of neonatal rat cardiomyocytes recorded in control condition, after application of 50 μM Ni^{2+} (4 min) and further application of 10 μM LPC (4 min) (B). Representative AP recorded in control condition, after application of 50 μM Ni^{2+} (4 min) and further application of 1 μM PMA (5 min) (C). Illustration of LPC (10 μM) effects with or without low concentration of Ni^{2+} (50 μM) on the beating rate (D), the maximum upstroke velocity ($\dot{V}_{max}$) (E) and the maximum diastolic potential (MDP) (F). Illustration of PMA (1 μM) effects with or without Ni^{2+} (50 μM) on the beating rate (G), $\dot{V}_{max}$ (H) and MDP (I). The number of cells used in each group is indicated in parentheses. *$p<0.05$.

automaticity (Fig. 1C). Meanwhile, effects of PKC on $I_{Ca.L}$ are controversial; PKC activation resulted in an increase of $I_{Ca.L}$ [25], a decrease of $I_{Ca.L}$ [26], or no change of $I_{Ca.L}$ [27]. Some researchers demonstrated a biphasic effect of PKC activators on $I_{Ca.L}$, i.e., a decrease of $I_{Ca.L}$ was followed by an increase [28]. These conflicting results may be due to differences in experimental conditions, species and/or methods of measuring $I_{Ca.L}$ as described by Kamp and Hell [29]. Although we confirm the upregulation of $I_{Cav3.2}$ by PKCα isoform activation (Fig. 4), PKCα may not have any effects on $I_{Ca.L}$ based on the findings in Fig. 2 by use of neonatal rat cardiomyocytes combined with LPC application. In our experiments, PMA significantly increased the beating rate of cardiomyocytes at the physiological $[Ca^{2+}]_i$ condition, which is sufficient to activate $[Ca^{2+}]_i$-dependent isoform of PKC, i.e., PKCα, PKCβI and PKCβII [30]. Taken together, upregulation of $I_{Ca.L}$ by PKCβ is potentially suggested, although no document has confirmed the hypothesis yet.

4.2. Interaction of LPC with intracellular signal PKC

Effects of LPC on cardiac ion channels such as the inward rectifier K^+ channel and the voltage-dependent Na^+ channel were intensively studied [22]. Previous studies have revealed that LPC modifies the ionic

channels probably by a direct interaction into the sarcolemmal membrane with biophysical effects and/or by activating protein kinase C. In this study, a pan-PKC inhibitor chelerythrine completely abolished the effect of LPC on $I_{Cav3.2}$ (Fig. 4). Moreover, a full PKC activation by 1 μM PMA masked the modulation of $I_{Ca.T}$ by LPC (Figs. 5 and 6). It is therefore firmly suggested that effect of LPC on $I_{Ca.T}$ is involved in PKC activation. The PKC activity in HEK293 cells has been widely documented [30,31], giving rationale to explore effects of LPC on $Ca_V3.2$-HEK293 cells. LPC causes an increase in cytosolic $[Ca^{2+}]_i$ [2,4], which is required to activate conventional PKC (cPKC) [30]. In this context, LPC is expected to impact $I_{Ca.T}$ in a $[Ca^{2+}]_i$-dependent manner (Fig. 3), which is highly consistent with our findings that LPC augments $I_{Ca.T}$ only when $[Ca^{2+}]_i$ is sufficiently elevated ($pCa \leq 9$). Out of 11 PKC isoforms, α, βI, βII, δ, ε, ζ and λ have been identified in the heart [9-11], where only isoforms α, βI and βII are dependent on intracellular Ca^{2+} for activation (cPKC). Importantly, LPC is to potentiate the activation of α- and β-isoforms of PKC in vitro [30]. Based on these backgrounds, with a use of specific inhibitors of PKCα, PKCβI or PKCβII, we firstly demonstrate that PKCα but not PKCβI or PKCβII exerts an action to modulate $I_{Ca.T}$ in LPC application. We therefore speculate that LPC accelerates cardiomyocyte automaticity via activating PKCα in physiological and/or pathophysiological conditions.

4.3. PKC activation augments the T-type Ca^{2+} channel

T-type Ca^{2+} channel activity, like that of most ionic channels, can be modulated by hormones and neurotransmitters action through signaling intermediates such as calmodulin-dependent protein kinase II [32] and lipid derivatives such as arachidonic acid [33]. PKC is a ubiquitously expressed family of kinases that phosphorylate a myriad of target proteins including ion channels, thereby regulating cellular excitation processes [11]. Studies of PKC-dependent modulation of the native $I_{Ca.T}$ have yielded conflicting results: $I_{Ca.T}$ is to be upregulated, downregulated or unaffected by PKC [34-37]. For example, $I_{Ca.T}$ in cultured neonatal rat ventricular myocytes was stimulated by 10 nM endothelin-1 via PKC activation [34], whereas $I_{Ca.T}$ in rat dorsal root ganglion neurons was inhibited by 10 nM PMA [35]. Similar inhibition of $I_{Ca.T}$ by PKC activators has been reported in the clonal GH3 line of anterior pituitary cells [36]. Park et al. recently reported that a PKC activator PMA increased $I_{Cav3.1}$ and $I_{Cav3.2}$ in the heterologous expression system in *Xenopus* oocyte [37], which is consistent with our results. These contradictory findings on T-type Ca^{2+} channel modulation by PKC might be caused by differences in cell conditions including $[Ca^{2+}]_i$. In this regard, it is a great advantage to assess $I_{Ca.T}$ expressed in a mammalian cell line HEK293, where we strictly controlled the intracellular Ca^{2+} concentration from a pCa of 7 to a pCa of 11 (Fig. 3). To our knowledge, we first report an upregulation of cardiac $Ca_V3.2$ T-type Ca^{2+} channel by LPC which is mediated by PKCα activation, highly depending on $[Ca^{2+}]_i$ condition. By use of heterologous system, $Ca_V3.2$ but not $Ca_V3.1$ channel was modulated by LPC, which is consistent with our results on action potential automaticity in Fig. 1. More importantly, previous reports on abnormal expression or remodeling of the T-type Ca^{2+} channel demonstrated the specific expression of the $Ca_V3.2$ channel in hypertrophied or failing cardiomyocytes [13]. In this content, it is expected that modulation of $I_{Cav3.2}$ by LPC exerts more intense effects on myocytes in pathological conditions in the heart. Because intracellular and extracellular accumulation of LPC in myocytes is caused by ischemia-associated elevation of cellular Ca^{2+} concentration, LPC may act as a potent stimulator of atrial and ventricular arrhythmias by activating remodeling $I_{Ca.T}$ excessively in the ischemic condition.

In conclusion, the present study indicates that intracellular signal PKCα activation by LPC upregulates the cardiac $I_{Ca.T}$ in physiological or higher $[Ca^{2+}]_i$ condition, which may accelerate the pathophysiological cardiac automaticity and trigger tachyarrhythmias as a novel ischemia-related mechanism.

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